

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Listing of the claims showing amendments.

Claims 1-59 (Canceled).

60. (Currently Amended) A method for increasing sensitivity and specificity of a one-tube RT-PCR method, comprising ~~the steps of~~:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding a PCR reagent composition containing a PCR primer set and a thermostable DNA polymerase to the reaction mixture; and
- (c) conducting a PCR amplification on the reaction mixture.

61. (Original) The method of claim 60, wherein prior to the PCR amplification, the PCR reagent composition is separated from the reaction mixture in a reaction vessel by a physical barrier which is removed prior to or during the first cycle of the PCR reaction, thereby adding the PCR reagent composition to the reaction mixture.

Claim 62. (Canceled).

63. (Original) The method of claim 60, wherein the reverse transcription reaction is conducted for about 2 minutes.

64. (Original) The method of claim 60, wherein the RT-PCR method is performed in an automated system and the reagents for the RT-PCR method are stored in a cartridge having a plurality of compartments in which the reagents are stored prior to use in the RT-PCR method, wherein the automated system adds the reagents to a reaction vessel from the cartridge according to a programmed sequence.

Claims 65-77 (Canceled).

78. (Currently Amended) An intraoperative PCR diagnostic method comprising ~~the steps of~~:

- (a) obtaining a tissue sample from a patient in an operation; and
- (b) determining by a PCR method performed during the operation if expression of an indicator transcript exceeds a threshold level.

79. (Currently Amended) The method of claim 78, further comprising ~~the step of~~ continuing the operation in a manner dictated by results of the determining step.

80. (Previously Presented) The method of claim 78, wherein the tissue sample is a tumor biopsy and the PCR method is specific to an indicator transcript that, when the indicator transcript is overexpressed, is indicative of a malignancy.

81. (Previously Presented) The method of claim 80, wherein the indicator transcript is a carcinoembryonic antigen transcript.

82. (Currently Amended) The method of claim 78, wherein the PCR method is a multiplex PCR method, comprising ~~the step of~~ conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages.

83. (Currently Amended) The method of claim 78, wherein the PCR method is a multiplex PCR method, comprising ~~the step of~~ conducting a PCR amplification on a PCR reaction mixture in a first stage and a second stage, the reaction mixture comprising a DNA sample, a first primer set having a first effective T_m and a second primer set having a second effective T_m different from the first effective T_m , each amplification stage comprising one or more PCR cycles, each PCR cycle comprising a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a different temperature ~~[[as]]~~than the annealing step of the second amplification stage to modulate the relative rate of production of a first amplicon by the first primer set and a second amplicon by the second primer set during the first and second amplification stages.

84. (Currently Amended) The method of claim 78, wherein the PCR method is a PCR method, comprising ~~the step of~~ conducting a PCR amplification, the PCR amplification comprising a plurality of PCR cycles, on a PCR reaction mixture comprising a nucleic acid sample, a primer set in which the concentration of each of the primers of the primer set is at least about 400 nM, each PCR cycle comprising a denaturing step, an annealing step and an elongation step which may be conducted ~~concurrently with~~ at the same temperature as the annealing step, wherein the PCR amplification produces one of a β -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a tyrosinase-specific amplicon and a carcinoembryonic antigen-specific amplicon.

85. (Currently Amended) The method of claim 78, wherein the PCR method is an RT-PCR method, comprising ~~the steps of~~:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding to the reaction mixture a first primer set having a first effective T_m , a second primer set having a second effective T_m different from the first effective T_m and a thermostable DNA polymerase; and
- (c) conducting a PCR amplification on the reaction mixture in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step of about 1 second or less, an annealing step of less than about 10 seconds and an elongation step of less than about 10 seconds that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a lower temperature than the annealing step of the second amplification stage to modulate the relative rate of amplification of a first target sequence by the first primer set and a second target sequence by the second primer set during the first and second amplification stages,

wherein the first target sequence is expected to be at least about 30-fold more prevalent in the DNA sample than the second target sequence.

86. (Currently Amended) The method of claim 78, wherein the PCR method is an RT-PCR method comprising ~~the steps of~~:

- (a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture; and
- (b) conducting a PCR reaction on the reaction mixture.

Claims 87-104 (Canceled).

105. (New) A multiplex PCR method, comprising conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages, wherein one of the first PCR primer set and the second PCR primer set produce one of a β -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a carcinoembryonic antigen-specific amplicon and a tyrosinase-specific amplicon.

106. (New) The method of claim 105, wherein one of the first PCR primer set and the second PCR primer set includes a primer comprising the sequence of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.

107. (New) The method of claim 105, wherein the first primer set produces a β -glucuronidase-specific amplicon and the second primer set produces a carcinoembryonic antigen-specific amplicon, the T_m of the first primer set being about 1°C lower than the T_m of the second primer set and the annealing temperature for the PCR amplification of the first amplification stage is about 1°C lower than the annealing temperature for the PCR amplification of the second amplification stage.

108. (New) The method of claim 105, wherein the first PCR primer set consists of SEQ ID NOS: 16 and 17, and the second PCR primer set consists of SEQ ID NOS: 6 and 7, the annealing temperature for the PCR amplification of the first amplification stage is the equivalent of about 53°C and the annealing temperature for the PCR amplification of the second amplification stage is the equivalent of about 64°C, based on initial primer concentration of both PCR primer sets of 400 nM/L and an effective T_m for the first PCR primer set of about 50°C and an effective T_m for the second PCR primer set of about 60°C.

109. (New) A multiplex PCR method, comprising conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages, wherein the amplification stages include one or more quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon.

110. (New) The method of claim 109, wherein the quantitative PCR reactions are fluorescent 5' nuclease assays.

111. (New) The method of claim 109, wherein the fluorescent reporter is a molecular beacon.

112. (New) A multiplex PCR method, comprising conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages, wherein the denaturation step for one or more cycles is about 1 second or less.

113. (New) The method of claim 112, wherein the denaturation step for each cycle is about 1 second or less.

114. (New) A multiplex PCR method, comprising conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that is conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages.

115. (New) A multiplex PCR method, comprising conducting a PCR amplification on a PCR reaction mixture in a first stage and a second stage, the reaction mixture comprising a DNA sample, a first primer set having a first effective T_m and a second primer set having a second effective T_m different from the first effective T_m , each amplification stage comprising one or more PCR cycles, each PCR cycle comprising a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a greater temperature than the annealing step of the second amplification stage to modulate the relative rate of production of a first amplicon by the first primer set and a second amplicon by the second primer set during the first and second amplification stages.

116. (New) An RT-PCR method comprising:

(a) conducting a reverse transcription reaction for less than about 10 minutes on an RNA sample in a reaction mixture; and

(b) conducting a PCR reaction on the reaction mixture.

117. (New) The method of claim 116, wherein the reverse transcription reaction is conducted for about 2 minutes.

118. (New) The method of claim 116, wherein the reverse transcription reaction is conducted prior to the first amplification stage, and prior to the addition of one of PCR primers and a thermostable DNA polymerase to the reaction mixture, to produce DNA in the DNA sample of the reaction mixture.

119. (New) A multiplex PCR method, comprising conducting a PCR amplification on a DNA sample in a PCR reaction mixture, wherein the PCR amplification is conducted in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the PCR amplification of the second amplification stage is conducted under different reaction conditions than the PCR amplification of the first amplification stage to modulate the relative rate of production of a first amplicon by a first primer set and a second amplicon by a second primer set during the first and second amplification stages, wherein one or both primers of the second primer set do not anneal to an amplicon product produced by the first primer set in the PCR amplification.

120. (New) The method of claim 119, wherein the second primer set is added to the reaction mixture at the beginning of the second amplification stage.

121. (New) The method of claim 119, wherein one of the first PCR primer set and the second PCR primer set produce one of a β -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a carcinoembryonic antigen-specific amplicon and a tyrosinase-specific amplicon.

122. (New) The method of claim 121, wherein one of the first PCR primer set and the second PCR primer set includes a primer comprising the sequence of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.

123. (New) The method of claim 121, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.

124. (New) The method of claim 119, wherein the reaction mixture comprises a DNA sample, the first primer set having a first effective T_m and the second primer set having a second effective T_m different from the first effective T_m , wherein the annealing step of the first amplification stage is conducted at a different temperature than the annealing step of the second amplification stage.

125. (New) The method of claim 124, wherein the annealing step and the elongation step in a PCR cycle of at least one of the first amplification stage and the second amplification stage are conducted at the same temperature.

126. (New) The method of claim 124, wherein the annealing step and the elongation step in a PCR cycle of at least one of the first amplification stage and the second amplification stage are conducted at different temperatures.

127. (New) The method of claim 124, wherein the first primer set produces a β -glucuronidase-specific amplicon and the second primer set produces a carcinoembryonic antigen-specific amplicon, the T_m of the first primer set being about 1°C lower than the T_m of the second primer set and the annealing temperature for the PCR amplification of the first amplification stage is about 1°C lower than the annealing temperature for the PCR amplification of the second amplification stage.

128. (New) The method of claim 126, wherein the first PCR primer set consists of SEQ ID NOS: 16 and 17, and the second PCR primer set consists of SEQ ID NOS: 6 and 7, the annealing temperature for the PCR amplification of the first amplification stage is the equivalent of about 53°C and the annealing temperature for the PCR amplification of the second amplification stage is the equivalent of about 64°C , based on initial primer concentration of both PCR primer sets of 400 nM/L and an effective T_m for the first PCR primer set of about 50°C and an effective T_m for the second PCR primer set of about 60°C .

129. (New) The method of claim 119, wherein the denaturation step for each cycle is about 1 second.

130. (New) The method of claim 119, wherein the denaturation step for each cycle is less than about 1 second.

131. (New) The method of claim 119, further comprising conducting a reverse transcription reaction on an RNA sample prior to the first amplification stage, and prior to the addition of PCR primers to the reaction mixture, to produce the DNA of the DNA sample of the reaction mixture.

132. (New) The method of claim 131, wherein the reverse transcription reaction is conducted for less than about 10 minutes.

133. (New) The method of claim 131, wherein the reverse transcription reaction is conducted for about 2 minutes.
134. (New) The method of claim 131, wherein one or both of an Internal Positive Control RNA and an Internal Positive Control DNA is added to the reverse transcription reaction.
135. (New) The method of claim 119, wherein an Internal Positive Control DNA is added to the PCR reaction mixture.
136. (New) The method of claim 135, wherein the Internal Positive Control DNA comprises the sequence of one of SEQ ID NOS 23-25.
137. (New) The method of claim 135, wherein the Internal Positive Control DNA contains one or more uracil residues.
138. (New) The method of claim 119, wherein the amplification stages include quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon.
139. (New) The method of claim 138, wherein the quantitative PCR reactions are fluorescent 5' nuclease assays.
140. (New) The method of claim 138, wherein the fluorescent reporter is a molecular beacon.
141. (New) The method of claim 119, wherein one or more reagents for the reaction mixture are provided for use in the reaction mixture in a cartridge suitably configured for use in an automated system.
142. (New) The method of claim 141, wherein the cartridge is disposable after a single use.
143. (New) The method of claim 141, wherein the cartridge contains additional reagents or mechanical components compartmentalized separately or together with the reagents for the reaction mixture, the additional reagents or mechanical components adapted for one of cell or tissue lysis, RNA purification and reverse transcription.
144. (New) The method of claim 141, wherein the amplification stages include quantitative PCR reactions using a fluorescent reporter to indicate accumulation of a specific amplicon and the automated system automatically shifts the PCR reaction from the first amplification stage to the second amplification stage when the fluorescent reporter accumulates in the reaction mixture to a threshold level.

145. (New) The method of claim 119, wherein the first and second stages are conducted sequentially in the same reaction vessel.

146. (New) The method of claim 119, wherein there is expected to be at least about a 30-100-fold difference in the number of target sequences of the first primer set and of the second primer set in the DNA sample.

147. (New) A multiplex PCR method, comprising conducting a PCR amplification on a PCR reaction mixture in a first stage and a second stage, the reaction mixture comprising a DNA sample, a first primer set having a first effective T_m and a second primer set having a second effective T_m different from the first effective T_m , each amplification stage comprising one or more PCR cycles, each PCR cycle comprising a denaturing step, an annealing step and an elongation step that may be conducted at the same temperature as the annealing step, wherein the annealing step of the first amplification stage is conducted at a different temperature than the annealing step of the second amplification stage to modulate the relative rate of production of a first amplicon by the first primer set and a second amplicon by the second primer set during the first and second amplification stages, wherein one or both primers of the second primer set do not anneal to an amplicon product produced by the first primer set in the PCR amplification.

148. (New) The method of claim 147, wherein the annealing step and the elongation step in a PCR cycle of at least one of the first amplification stage and the second amplification stage are conducted at the same temperature.

149. (New) The method of claim 147, wherein the annealing step and the elongation step in PCR cycles of at least one of the first amplification stage and the second amplification stage are conducted at different temperatures.

150. (New) The method of claim 147, wherein the effective T_m of the first primer set and the effective T_m of the second primer set differ by at least about 5°C.

151. (New) The method of claim 147, wherein one of the first PCR primer set and the second PCR primer set produce one of a β -glucuronidase-specific amplicon, an 18SrRNA-specific amplicon, a carcinoembryonic antigen-specific amplicon and a tyrosinase-specific amplicon.

152. (New) The method of claim 151, wherein one of the first PCR primer set and the second PCR primer set includes a primer comprising the sequence of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.

153. (New) The method of claim 151, wherein one of the first PCR primer set and the second PCR primer set includes a primer consisting of one of SEQ ID NOS: 3, 4, 6, 7, 11, 12, 13, 14, 16, 17, 19 and 20.

154. (New) The method of claim 151, wherein the annealing step of the first amplification stage is conducted at a temperature greater than the annealing step of the second amplification stage.

155 (New) The method of claim 154, wherein one of the first and second primer sets produces one of a β -glucuronidase-specific amplicon, a carcinoembryonic antigen-specific amplicon, an 18SrRNA amplicon and a tyrosinase amplicon.

156. (New) The method of claim 154, wherein the first primer set produces a β -glucuronidase-specific amplicon and the second primer set produces a carcinoembryonic antigen-specific amplicon.

157. (New) The method of claim 147, wherein the annealing step of the first amplification stage is conducted at a temperature less than the annealing step of the second amplification stage.

158. (New) The method of claim 147, further comprising conducting a reverse transcription reaction on an RNA sample prior to the first amplification stage, and prior to the addition of one of PCR primers and a thermostable DNA polymerase to the reaction mixture, to produce DNA in the DNA sample of the reaction mixture.

159. (New) The method of claim 158, wherein the reverse transcription reaction is conducted for less than about 10 minutes.

160. (New) The method of claim 158, wherein the reverse transcription reaction is conducted for about 2 minutes.

161. (New) The method of claim 158, wherein one or both of an Internal Positive Control RNA and an Internal Positive Control DNA is added to the reverse transcription reaction.

162. (New) The method of claim 147, wherein an Internal Positive Control DNA is added to the PCR reaction mixture.

163. (New) The method of claim 147, wherein the Internal Positive Control RNA comprises the sequence of one of SEQ ID NOS 23-25.

164. (New) The method of claim 147, wherein one or more reagents for the reaction mixture are provided for use in the reaction mixture in a cartridge suitably configured for use in an automated system.

165. (New) The method of claim 164, wherein the cartridge is disposable after a single use.

166. (New) The method of claim 164, wherein the cartridge contains additional reagents or mechanical components compartmentalized separately or together with the reagents for the reaction mixture, the additional reagents or mechanical components adapted for one of cell or tissue lysis, RNA purification and reverse transcription.

167. (New) The method of claim 147, wherein the amplification stages include one or more quantitative PCR reaction using a fluorescent reporter to indicate accumulation of a specific amplicon.

168. (New) The method of claim 167, wherein the quantitative PCR reactions are fluorescent 5' nuclease assays.

169. (New) The method of claim 167, wherein the fluorescent reporter is a molecular beacon.

170. (New) An RT-PCR method, comprising:

- (a) conducting a reverse transcription reaction on an RNA sample in a reaction mixture to produce a DNA sample;
- (b) adding to the reaction mixture a first primer set having a first effective T_m , a second primer set having a second effective T_m different from the first effective T_m and a thermostable DNA polymerase; and
- (c) conducting a PCR amplification on the reaction mixture in a first amplification stage and a second amplification stage, each amplification stage comprising one or more PCR cycles and each PCR cycle comprises a denaturing step, an annealing step that may be conducted at the same temperature and the same time as the annealing step, wherein the annealing step of the first amplification stage is conducted at a lower temperature than the annealing step of the second amplification stage to modulate the relative rate of amplification of a first target sequence by the first primer set and a second target sequence by the second primer set during the first and second amplification stages,

wherein first target sequence is expected to be at least about 30-fold more prevalent in the DNA sample than the second target sequence, and wherein:

- d) the RT reaction is conducted for less than about 10 minutes; or
- e) the denaturing step is conducted for about 1 second or less.

171. (New) The method of claim 170, wherein the RT reaction is conducted for less than about 10 minutes, and, for one or both of the first and second amplification stages, the denaturing step is conducted for about 1 second or less, the annealing step is conducted for less than about 10 seconds and the elongation step is conducted for less than about 10 seconds.